Biotechnology & Industrial Microbiology

Microbiology & Immunology Dept.

Lecture 4

Continue..

Examples of important products produced by fermentation

IV. Health-care products

- The major health-care products derived from microbial fermentations and/or biotransformation are alkaloids, steroids, toxins, vaccines, vitamins and a wide variety of mammalian proteins and peptides (insulin, interferons, human growth hormone and monoclonal antibodies).

- Most **antibiotics** are secondary metabolites. The best known and probably the most medically important antibiotics are the β-lactams (penicillins and cephalosporins) along with aminoglycosides, such as (streptomycin).

1. Antibiotics

Antibiotic	*Producing microorganism*
Cephalosporin	*Cephalosporium acrimonium*
Chloramphenicol	*Streptomyces venezuelae*
Erythromycin	*Streptomyces erythreus*
Griseofulvin	*Penicillium griseofulvin*
Penicillin	*Penicillium chrysogenum*
Streptomycin	*Streptomyces griseus*
Tetracycline	*Streptomyces aureofaciens*
Gentamicin	*Micromonospora purpurea*

A. PENICILLIN

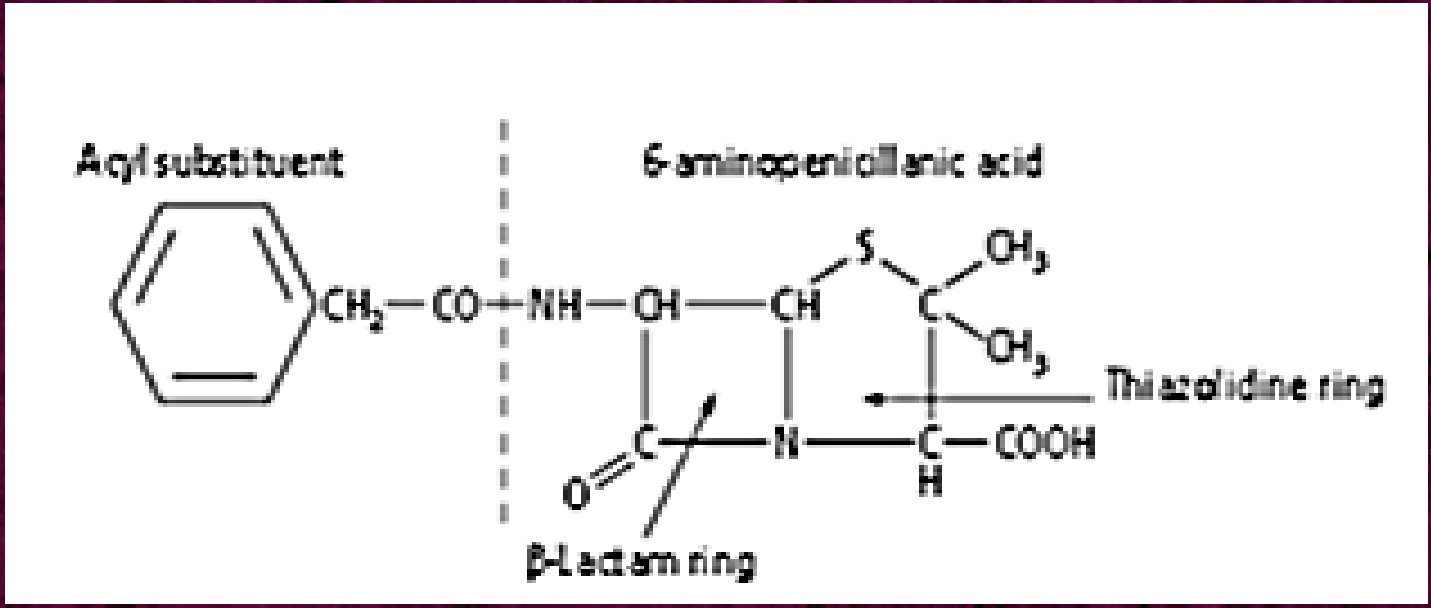
Thanks to work by Alexander Fleming (1881-1955), Howard Florey (1898-1968) and Ernst Chain (1906-1979), penicillin was first produced on a large scale for human use in 1943. At this time, the development of a pill that could reliably kill bacteria was a remarkable development and many lives were saved during World War II because this medication was available.

A. Fleming

E. Chain

H. Florey

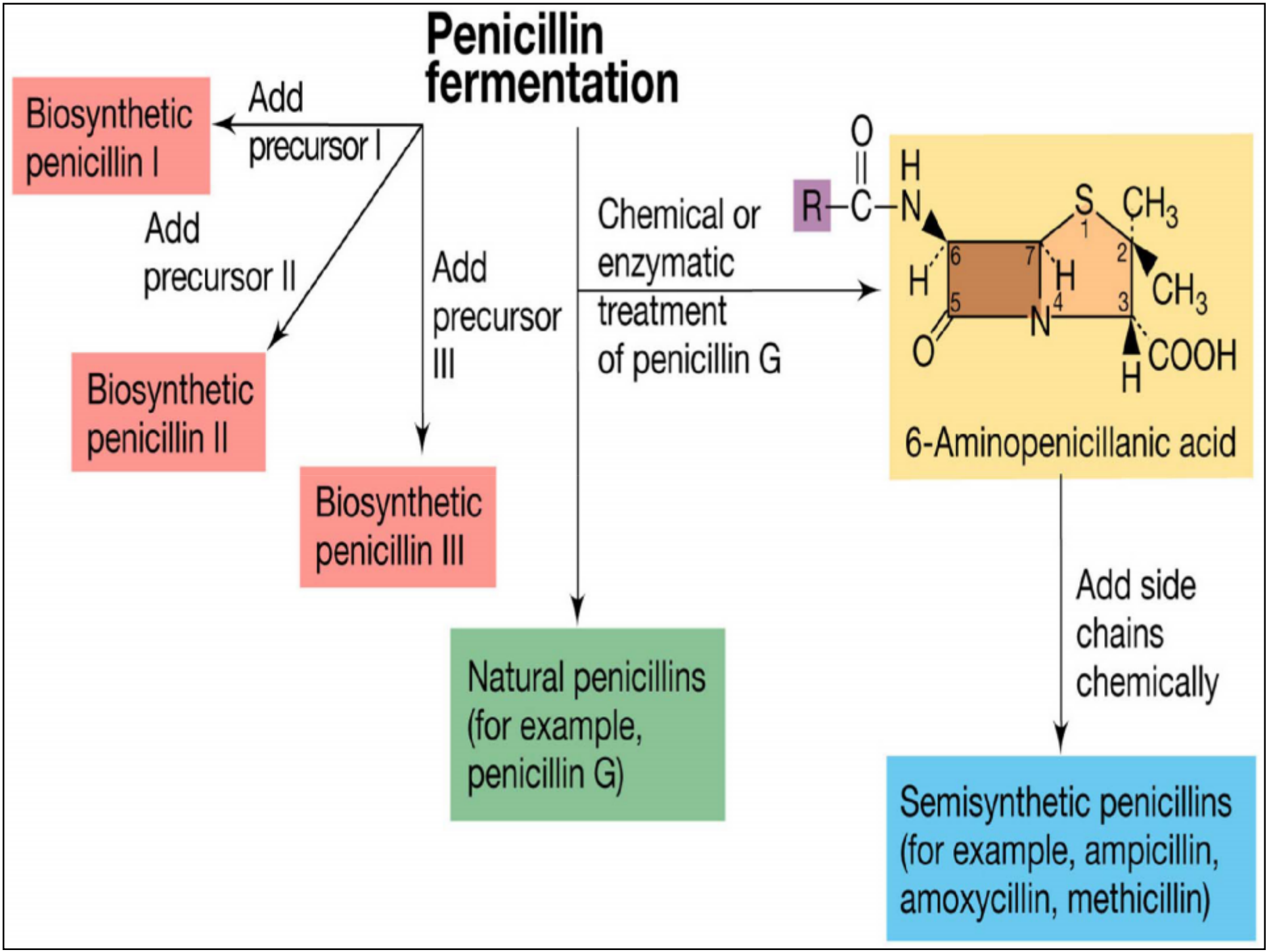
- The basic structure of the penicillins is **6-aminopenicillanic acid (6-APA)**, composed of a **thiazolidine ring** fused with a β-**lactam ring** whose 6-amino position carries a variety of **acyl** substituents.



- In the absence of added side-chain precursors to the fermentation medium of *P. notatum* or *P. chrysogenum*, a **mixture of natural penicillins** is obtained from culture filtrates, notably penicillin G (benzyl penicillin) and the more acid-resistant penicillin V (phenoxymethyl penicillin). These penicillins are most active against Gram positive bacteria.

- However, an expanded role for the penicillins came from the discovery that different **biosynthetic penicillins** can be formed by the addition of **side-chain precursors** to the fermentation medium and that natural penicillins can be modified chemically to produce compounds with improved characteristics.

- Most penicillins are now **semisynthetic**, produced by the chemical modification of natural penicillins, obtained by fermentation using strains of *P. chrysogenum*.
- Modification is achieved by **removing their natural acyl group**, leaving 6-APA, to which other acyl groups can be added to confer new properties.
- These semisynthetic penicillins, such as methicillin, carbenicillin and ampicillin, exhibit various improvements, including resistance to stomach acids to allow oral administration, a degree of resistance to penicillinase and an extended range of activity against some Gram-negative bacteria.


Penicillin fermentation
Biosynthetic penicillin I
Add precursor I
Add precursor II
Biosynthetic penicillin II
Add precursor III
Biosynthetic penicillin III
Chemical or enzymatic treatment of penicillin G
Natural penicillins (for example, penicillin G)
6-Aminopenicillanic acid
Add side chains chemically
Semisynthetic penicillins (for example, ampicillin, amoxycillin, methicillin)

Commercial production of penicillin

Condition	source
Operating mode	Submersion STR fed batch (40,000- 200,000 L)
Organism	*P. chrysogenum*
Temperature	25–27° C
pH	6.5–7.7 by calcium carbonate (1%, w/v) and a phosphate buffer to neutralize the medium
carbon sources	glucose, lactose ????? sucrose, ethanol and vegetable oils
Nitrogen sources	Corn steep liquor
others	Ammonia, mineral salts and specific side-chain precursors, e.g. phenyl acetic acid or phenoxyacetic acid, may also be added (must be fed continuously at non-inhibitory concentrations)
Oxygen level	25-60 mmol/L/h

Commercial production of penicillin

Phase I: Growth phase (vegetative phase)

- Adding lyophilized spores to a small fermenter at a concentration of $5x10^3$ spores/ml.
- Glucose as carbon source ????
- Fungal mycelium is grown up through one or two further stages until there is sufficient to inoculate the production fermenter.
- This high growth rate is maintained for the first 2 days.

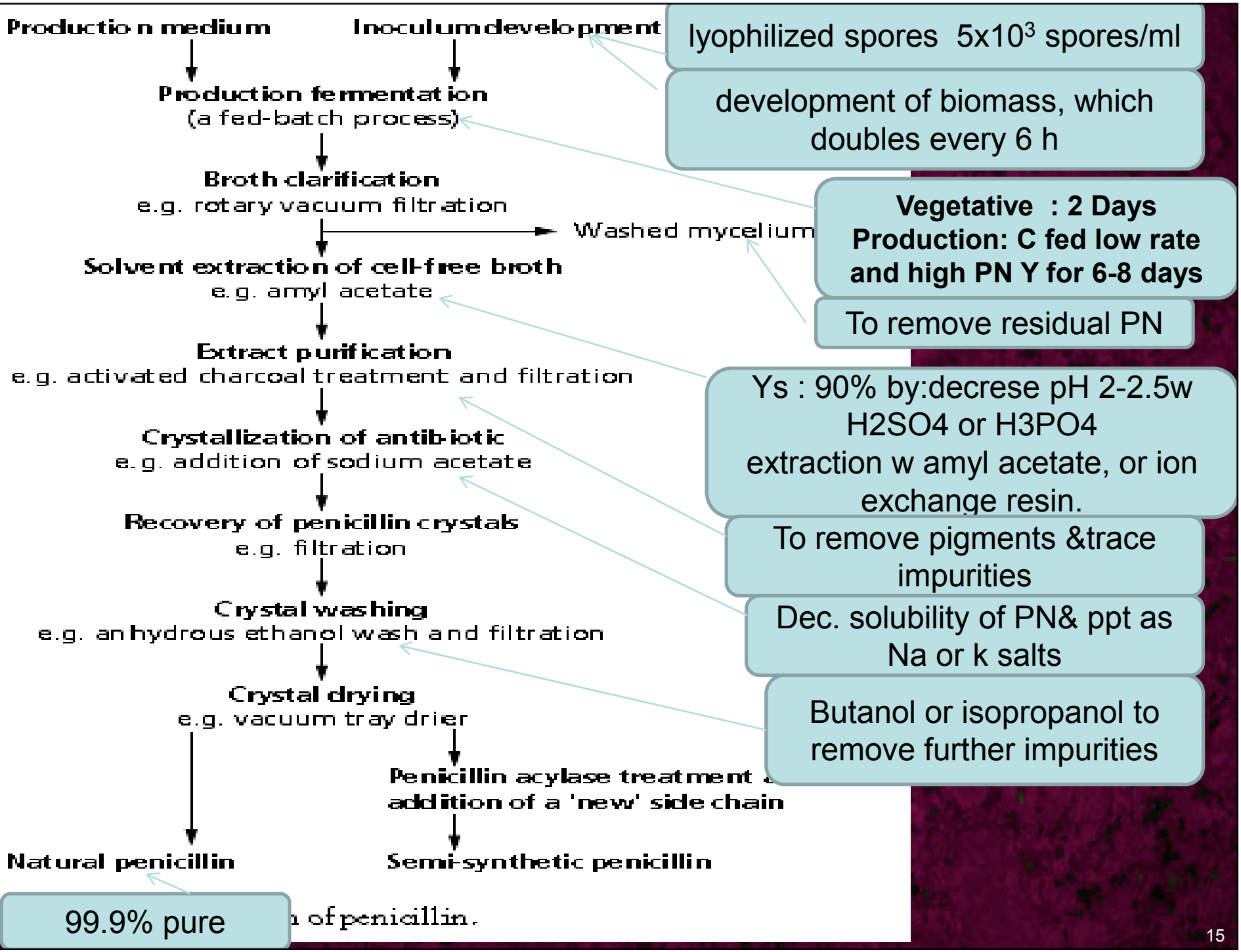
Phase II: Production phase

- The mycelium is transferred to another medium containing lactose as a carbon source (lactose) which is fed at a low rate.
- Penicillin production increases. This continues for a further 6–8 days.
- Penicillin is excreted into the medium and is recovered at the end of this phase otherwise it starts to degrade.

Phase III: penicillin recovery

- Penicillin recovery by removal of mycelium using rotary vacuum filters.
- Recovered mycelium is then **washed** to remove residual penicillin, prior to its use as animal feed or fertilizer.
- Antibiotic recovery is often by **solvent extraction** of the cell-free medium, which gives yields of up to 90%. This involves reducing the pH of the filtered medium to 2.0–2.5 by addition of sulphuric or phosphoric acid, followed by a rapid extraction at 0–3° C using **amyl acetate**. The low temperature is necessary to reduce damage to penicillin due to the low pH.
- Alternatively, ion-pair extraction may be used at pH 5–7, in which range penicillin is stable.

- Any pigments and trace impurities are removed by treating with activated charcoal.
- The penicillin is then precipitated from the solvent by addition of sodium or potassium acetate and it precipitates as a sodium or potassium salt.
- Resultant penicillin crystals are separated by rotary vacuum filtration.
- Penicillin crystals are washed with a volatile solvent, to remove further impurities, then dried .


Production medium
Inoculum development
Production fermentation
(a fed-batch process)
Broth clarification
e.g. rotary vacuum filtration
Washed mycelium
Solvent extraction of cell-free broth
e.g. amyl acetate
Extract purification
e.g. activated charcoal treatment and filtration
Crystallization of antibiotic
e.g. addition of sodium acetate
Recovery of penicillin crystals
e.g. filtration
Crystal washing
e.g. anhydrous ethanol wash and filtration
Crystal drying
e.g. vacuum tray drier
Penicillin acylase treatment
addition of a 'new' side chain
Natural penicillin
Semi-synthetic penicillin
of penicillin.
lyophilized spores 5x10^3 spores/ml
development of biomass, which doubles every 6 h
Vegetative : 2 Days
Production: C fed low rate and high PN Y for 6-8 days
To remove residual PN
Ys : 90% by:decrese pH 2-2.5w H2SO4 or H3PO4
extraction w amyl acetate, or ion exchange resin.
To remove pigments &trace impurities
Dec. solubility of PN& ppt as Na or k salts
Butanol or isopropanol to remove further impurities
99.9% pure

Production of semisynthetic penicillins and cephalosporins

- It involves removal of the side chain of the base penicillin to form 6-APA.
- This is achieved by passage through a column of immobilized **penicillin acylase**, usually obtained from *Escherichia coli,* at neutral pH. Penicillin G, for example, is converted to 6-APA and phenylacetic acid. The 6-APA is then chemically acylated with an appropriate side chain to produce a semisynthetic penicillin.

- Yields of cephalosporins from direct fermentations are much lower than those for penicillins.
- Consequently, as 6-APA can also serve as a precursor of cephalosporins, it is often used as the starting material for their semisynthetic production. A base natural penicillin is converted to 6-APA, as described above, followed by its conversion to the preferred precursor, 7-amino deacetoxycephalosporic acid (7-ADCA), by ring expansion.
- A suitable side chain can then be readily attached.

B. STREPTOMYCIN

- It is the first antibiotic of aminoglcosides to be discovered.
- It is derived from ***Streptomyces griseus*.**
- Streptomycin is broad spectrum drug effective against both gram positive and gram negative bacteria. However, its major use is as an anti-TB drug.
- It inhibits protein synthesis.

- Streptomycin is manufactured by fermentation process. The process comprises three major phases :

1. Growth phase: Inoculum preparation.
2. Production phase: Fermentation.
3. Extraction, recovery and purification.

- Fermentation process is an **aerobic submerged type fermentation**

- First step is the preparation of inoculum from the stock culture spores of the strain. The inoculum is transferred to a germinator, where total quantity of biomass is increased.

- Second, this biomass is sent to the first of a series of fermentors in which medium has been introduced (Glucose, soybean meal, high agitation and aeration, PH 7.6-8, 28 ° C). Fermentation lasts for 10 days.

- Recovery of streptomycin:

1-The mycelium is separated by filtration.

2-Streptomycin is adsorbed by charcoal or ion exchange resin and then eluted with acid alcohol.

3- the antibiotic is precipitated with acetone and then filtered.

4-finally purified by chromatographic methods.

2. Steroid biotransformation

- Many steroids are now manufactured using a combination of **chemical and microbial transformation steps.**

- **Examples:**

Androgens: Testosterone.

Corticosteroids: Cortisone, hydrocortisone, and dexamethasone.

Oestrogens: Oestradiol and estrone.

Gestagens: Progesterone.

- These processes employ relatively cheap sterols as the starting materials. The microorganisms involved are mostly filamentous fungi or mycobacteria.

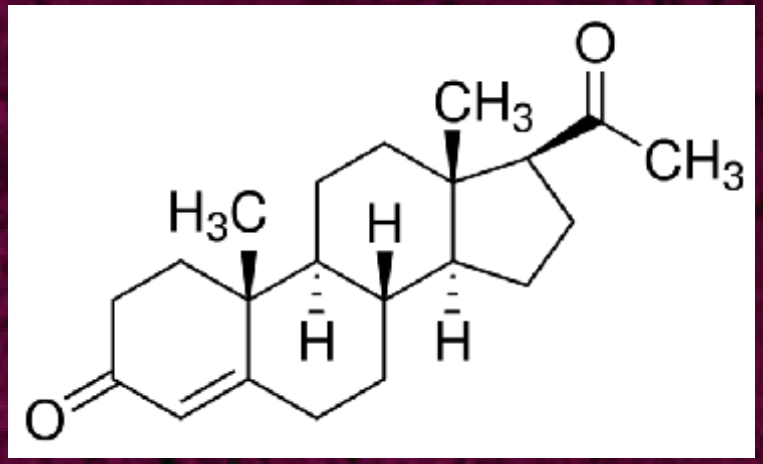
- They perform key reactions to modify the basic steroid structure, including hydroxylations at positions 11 and 17; various side-chain cleavages, hydrogenations and dehydrogenations; and ring expansions, from a five-membered to a six-membered ring.

Progesterone ***11 a-Hydroxylation*** 11a-Hydroxyprogesterone

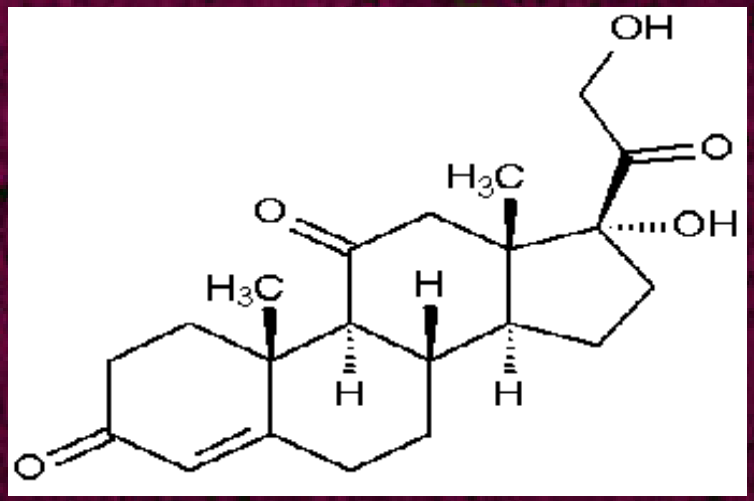
Rhizopus nigricans

chemically Hydrocortisone ***chemically*** Cortisone

Progesterone



Cortisone



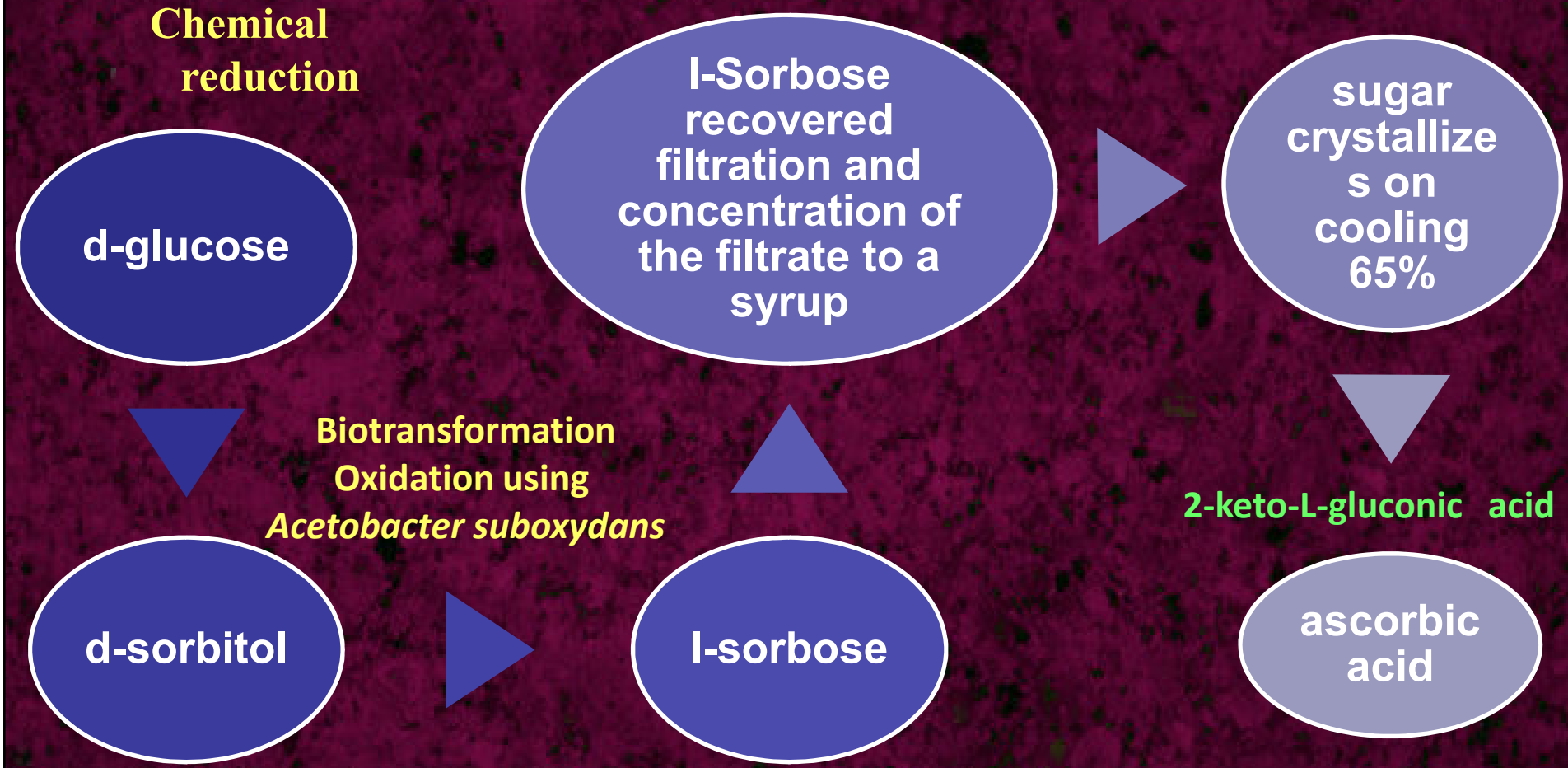
3- Vitamins

- Most vitamins were previously prepared from animal and plant tissues. However, microorganisms are now used as sources of a wide range of these important compounds, including thiamin (vitamin B1), riboflavin (vitamin B2), pyridoxine(vitamin B6), cobalamin (**vitamin B12**), biotin, folic acid, L-ascorbic acid (**vitamin C**), β-carotene (provitaminA), ergosterol (provitamin D2) and pantothenic acid.

- For the production of some vitamins:

1) Direct fermentation processes are operated, whereas for others,
2) Combined chemical and microbiological processes are employed.

Ascorbic acid (vitamin C)



- Annual production of ascorbic acid is now over 40000 tonnes. The established process involves chemical stages and a microbial biotransformation

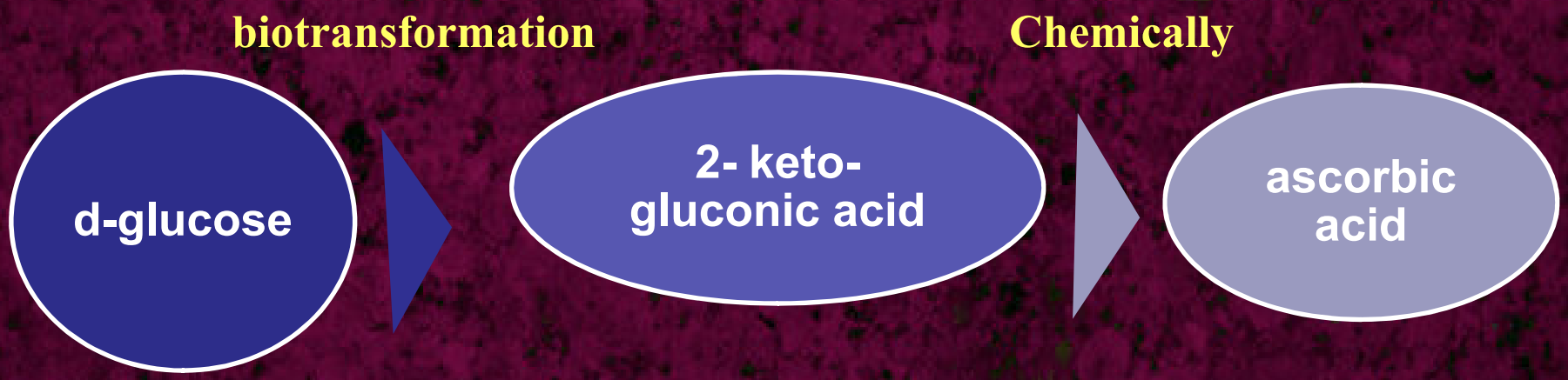
- Media for biotransformation step consist of glucose, yeast extract or corn steep liquor, a slight excess of calcium carbonate and 15-30% (w/v) d-sorbitol.

- The biotransformation is performed at 30° C under vigorous aeration and within 1–2 days a 90-95% conversion is achieved.
- Recent method ????

Ascorbic acid (vitamin C)

Using genetically Modified Microorganism (GMM) - *Erwinia*

This includes only two steps: Biotransformation & Chemical



Cobalamin (vitamin B12)

- Vitamin B12 is used as a food supplement and is particularly important in the treatment of pernicious anaemia.
- The two-phase industrial production process employs the bacteria *Propionibacterium shermanii*.

the first stage of the fermentation is conducted under anaerobic conditions in the absence of the B12 precursor, thereby preventing synthesis of the vitamin that would otherwise have a repressive effect. This leads to the accumulation of the intermediate, cobinamide

In the second phase, the culture is aerated and dimethylbenzimidazole is added to facilitate the conversion of cobinamide to vitamin B12. On purification it is isolated in the form of cyanocobalamin.

4- Bacterial vaccines

- Bacterial vaccines can be divided into two categories:

1- Living vaccines: composed of live attenuated strains of the parent virulent strain.

2- Inactivated vaccines: composed of whole killed bacterial cells, or a cell component or metabolic product (cell wall antigen, capsular antigens, toxin, etc.), which now may be products of recombinant DNA technology.

- Microbial protein **toxins** can serve as vaccines following their inactivation with formaldehyde or heat to form **toxoids**.

- Vaccination with an antigenic toxoid vaccine leads to the generation of **antitoxin** that neutralizes the pharmacological effect of active toxin. These vaccines have been successful against **Gram-positive** bacteria responsible for diphtheria, tetanus and several other diseases caused by Clostridium species. But they showed less activity against **Gram-negative** bacteria.

Vaccine production

- It requires highly controlled operating conditions and strict adherence to good manufacturing practices. Internal pressures **never exceed atmospheric** pressure, to reduce the risks of leakage, and **exhaust gases** from fermenters **must pass through sterilizing filters, incinerators** or both.
- Vaccine production normally involves the growth of bacterial cultures in sophisticated high-grade fermenters of usually no greater than 1000 L capacity

- The fermentations are designed for optimized yield of antigen (cells or cell components).

1-Fermentations for the production of vaccines based on whole cells aim to maximize biomass production.

- The microbial cells, inactivated or live, are then separated from the medium by **centrifugation**. For inactivated whole cell vaccines, downstream processing usually follows cell inactivation by **heat treatment or the addition of formaldehyde.**

2-For the production of vaccines based on toxins or surface antigens (cell wall or capsular components), the growth conditions are aimed at producing maximum levels of these specific cellular antigens.

- Excreted toxins and loosely bound surface antigens that are shed into the medium are purified from the clarified culture broth and the harvested cells are safely discarded.

Vaccine antigens for human immunization are highly purified.

Purification procedures may include

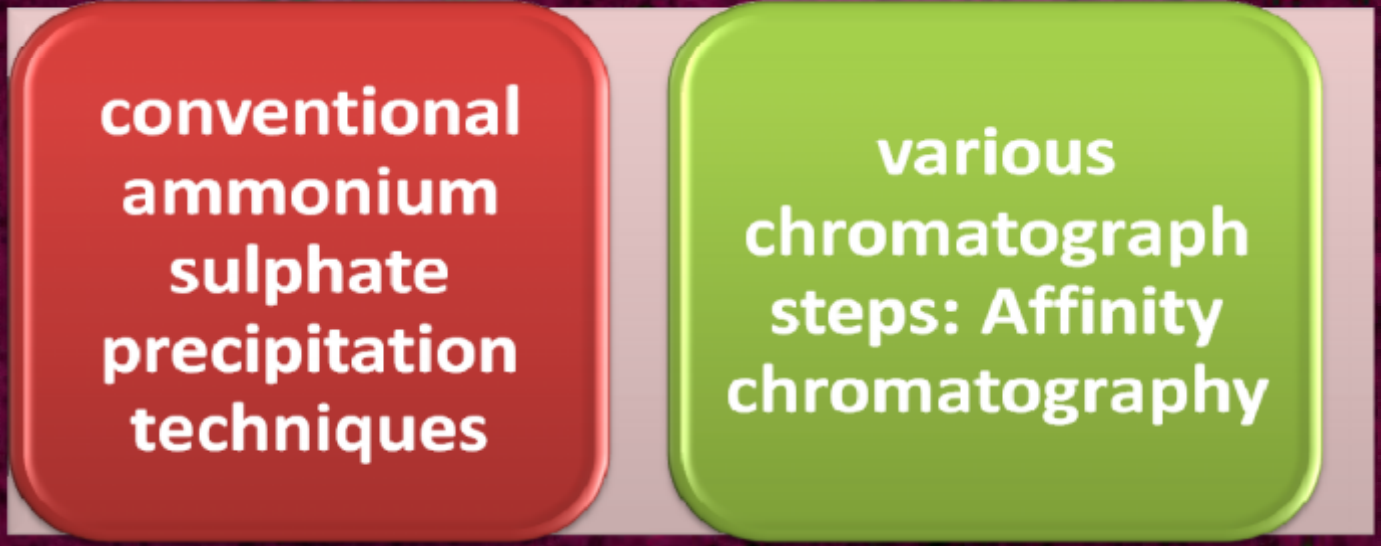


Table 11.5 Examples of recombinant proteins for medical use

Antibodies
tissue necrosis factor-α antibody (rheumatoid arthritis treatment)
Cancer and viral diseases
interferons
interleukins
tissue necrosis factors
transforming growth factors
Cardiovascular diseases
erythropoietin (boosts red blood cell proliferation)
hirudin (thrombin inhibitor from leech)
urokinase (thrombosis treatment)
tissue plasminogen activator (clot dissolution)
Hormones
human growth hormone
insulin
Neurological diseases
endorphins
nerve growth factors
neuropeptides
Vaccines (also see Table 11.4)
foot-and-mouth disease
hepatitis B
Wound healing and blood clotting factors
epidermal growth factor
fibroblast growth factor
clotting factor VIII (haemophilia treatment)

Lecture Summary

- Examples of important products produced by fermentation (Health care products)

Quiz

1- Complete the following statements:

1) Penicillin and streptomycin are produced by …… and …….. bacteria, respectively.

2) …… and …… are examples of vitamins produced by fermentation.

3) The microorganisms mostly involved in steroids biotransformation are ……. and .....

4) Bioremediation may be either …… or ……

5) ……. bacteria commonly used in fuels and industrial chemical production.

Thank you!!

Questions??